

Synergistic action of endothelin (ET)-1 on the activation of bronchial fibroblast isolated from normal and asthmatic subjects

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Summary. Bronchial subepithelial fibrosis is an histological characteristic of asthma. Cytokines and other mediators, such as PDGF-BB, TGF- β 1 and ET-1 found in the asthmatic submucosa can potentially activate a repair process that leads to fibroblast proliferation and collagen synthesis. The mechanisms of modulation of the repair process leading to extracellular matrix deposition are still to be documented. In this study, we assessed the *in vitro* proliferation and collagen synthesis of bronchial fibroblasts isolated from normal and asthmatic subjects in response to ET-1, platelet-derived growth factor (PDGF)-BB and transforming growth factor (TGF)- β 1 alone or in combination, in the presence or absence of dexamethasone. The combination of ET-1 with one of the other two growth factors, or the triple combination, significantly increased DNA synthesis and collagen production of bronchial fibroblasts isolated from both normal and asthmatic subjects, but the same growth factors used separately had no significant effect on the same parameters. These results suggest that the simultaneous presence of ET-1, PDGF-BB and TGF- β 1 in both normal and asthmatic subjects is necessary to activate bronchial fibroblast proliferation and collagen synthesis. As these mediators are present in the submucosa of the asthmatic bronchi, they could be responsible, at least in part, for the accumulation of collagen in the mucosa.

Keywords: collagen, asthma, bronchial fibroblast, endothelin

Introduction

The mechanisms by which symptomatic asthma and airway hyper-responsiveness develop are still unknown.

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However, airway inflammation and, more recently, bronchial structural changes have been implicated in the pathogenesis of asthma (Kay 1991; Bousquet *et al.* 1995). Histological features of asthma include airway wall oedema, inflammatory cell infiltrate of the airways mostly consisting of lymphocytes, mast cells and eosinophils (Jeffery *et al.* 1989; Djukanovic *et al.* 1990;

Rossi & Olivieri 1997) and airway remodelling such as subepithelial fibrosis, bronchial epithelium damage and smooth muscle cell changes (Brewster *et al.* 1990; Bousquet *et al.* 1995; Boulet *et al.* 1997). Airway subepithelial fibrosis results from the deposition, likely by activated fibroblasts, of interstitial collagens I, III and V, and fibronectin in the lamina reticularis of the basement membrane (Roche *et al.* 1989; Brewster 1990; Roche 1991; Chakir *et al.* 1996).

Cytokines produced by inflammatory cells can modulate fibroblast functions and extracellular matrix (ECM) deposition (Bousquet *et al.* 1992; Vignola *et al.* 1997). Transforming growth factor- β (TGF- β) and platelet derived growth factor (PDGF) are among the most potent cytokines affecting extracellular matrix component synthesis, fibroblast proliferation and structural cell phenotype in diseases such as pulmonary fibrosis and scleroderma (Gauldie *et al.* 1993; McWhirter *et al.* 1994). The level of TGF- β and its expression are increased in bronchoalveolar lavage fluid (BALF) and bronchial biopsies from asthmatic subjects (Redington *et al.* 1997; Vignola *et al.* 1997). PDGF is also present in BALF but its level does not seem to be increased in asthma compared to normal subjects (Chanez *et al.* 1995). Endothelin-1 (ET-1) is a potent bronchoconstrictor peptide secreted by the airway epithelium that has been implicated in asthma pathophysiology (Vittori *et al.* 1992). Elevated levels of ET-1 were recently found in the BALF and the plasma of asthmatic patients (Aoki *et al.* 1994; Redington *et al.* 1995). The concentrations of ET-1 in the plasma correlated with the severity of acute asthma and were inversely correlated with the ratio of forced expiratory volume in one second (FEV1) over forced vital capacity (FVC) (Aoki *et al.* 1994). ET-1 BALF levels were also shown to be inversely correlated with the percentage predicted FEV1 (Redington *et al.* 1995). ET-1 also has the potential to modulate airway smooth muscle contraction, mucus secretion, airway wall remodelling and fibroblast proliferation (Cambrey *et al.* 1994; Hay *et al.* 1996). Furthermore, glucocorticoid treatment can decrease the release of ET-1 in BALF of subjects with symptomatic asthma (Mattoli *et al.* 1991). It has been hypothesized that ET-1 alone or combined with other stimuli could activate bronchial fibroblasts from asthmatic airways to proliferate, synthesize collagen and with time, contribute to the subepithelial fibrosis (Springall *et al.* 1991; Hay *et al.* 1996). Data obtained with dermal or lung fibroblasts cannot be directly extrapolated to bronchial fibroblasts, as it is well recognized that cells of different origins or immortalized cells do not respond similarly to a specific stimulus (Durant *et al.* 1986; Verhofstad *et al.* 1998).

We have previously developed a method to isolate fibroblasts from bronchial biopsies of normal and asthmatic subjects (Dubé *et al.* 1997). In a previous study, we showed that bronchial fibroblasts isolated from asthmatic subjects did not demonstrate a high proliferative phenotype and that neither TGF- β 1 nor PDGF-BB alone was responsible for an increased proliferation of these cells (Dubé *et al.* 1998). The same study also showed that these fibroblasts did not spontaneously produce increased amounts of collagen. This led us to believe that a specific cytokine or mediator, or combination of these substances could promote the activation of bronchial fibroblasts in asthma. On the other hand, glucocorticoids have been shown to modulate fibroblast proliferation, collagen deposition and expression of collagenases and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (Durant *et al.* 1986; Clark *et al.* 1987; Mintz & Mann 1990; Hein 1993; Bizot-Foulon *et al.* 1995; Wangoo *et al.* 1997).

In an effort to define the mechanisms responsible for the mucosal collagen deposition encountered in asthma, we hypothesized that ET-1 alone or in combination with TGF- β 1 and/or PDGF-BB could activate bronchial fibroblasts from the airways of normal and asthmatic subjects to proliferate and synthesize procollagen I and III. We also evaluated the modulation of these responses by dexamethasone.

Methods

Subjects and fibroblast culture

Bronchial biopsies of seven asthmatic and five normal subjects were obtained by bronchoscopy or surgical specimens as described previously (Dubé *et al.* 1998). Fibroblasts were isolated from bronchial biopsies according to a method developed in our laboratory (Goulet *et al.* 1996) and culture purity was determined by immunofluorescence against vimentin and human fibroblast antigen (Ab-1 from Calbiochem, San Diego, CA, USA) (Dubé *et al.* 1998). Briefly, the bronchial biopsies were digested overnight at 4 °C using collagenase H (Boehringer Mannheim, Laval, Québec, Canada) in Dulbecco's modified Eagle's medium (DME) with 10 mM CaCl₂. The digestion product was seeded in 50 mm Petri dishes using DME with 10% fetal calf serum (FCS). Absence of ET-1, GM-CSF, IL-6, IL-8 and TGF- β 1 in FCS was confirmed by ELISA kits purchased from Intermedico (Markham, Ontario, Canada) for TGF- β 1 and from R & D Systems Inc (Minneapolis, MN, USA) for the others. Bronchial fibroblasts were also shown not to produce ET-1 in culture using the same ELISA kit as

described above. Test sensitivity was of 1.5 pg/ml for ET-1, 2.8 pg/ml for GM-CSF, 0.7 pg/l for IL-6, 10 pg/ml for IL-8 and 0.05 ng/ml for TGF- β 1.

Proliferative response of bronchial fibroblasts to ET-1, TGF- β 1 and PDGF-BB alone or in combination and modulation by dexamethasone

DNA synthesis measurements by ^3H -thymidine incorporation were used to measure bronchial fibroblast proliferation as previously described (Dubé *et al.* 1998). Fibroblasts for these experiments were used at passage three to five. Normal and asthmatic bronchial fibroblasts were cultured to 80–90% confluency. Cells were trypsinized and washed in DMEM with 10% FCS, 25 ng/ml Fungizone® from Sigma chemicals (St. Louis, MO, USA) and 20 units/ml gentamycin from ICN Biomedicals Inc (Aurora, OH, USA). Flat-bottomed 96 well plates were seeded at a density of 1×10^4 cells/well in DMEM with 0.5% FCS. Low serum conditions (0.5% FCS) were used to decrease serum mitogenic effects. Cells were then incubated for 48 h at 37 °C and 5% CO_2 in eight different culture conditions with or without 10^{-7} M dexamethasone from Sabex (Boucherville, Québec, Canada): (1) control: DMEM containing 0.5% FCS alone or with addition of either: (2) 5 ng/ml TGF- β 1 from Genzyme (Cambridge, MA, USA); and (3) 5 ng/ml PDGF-BB from Genzyme (Cambridge, MA, USA); (4) 10 nM ET-1 from Boehringer Mannheim (Laval, Québec, Canada); (5) both PDGF-BB and TGF- β 1; (6) both ET-1 and TGF- β 1; (7) both ET-1 and PDGF-BB; or (8) all three ET-1, PDGF-BB and TGF- β 1. After the initial 48 h, ^3H -thymidine (1 μCi /well) from ICN (Aurora, OH, USA) was added to the culture for another 24 h. At the end of this period, the medium was removed and the cells were trypsinized and collected on a glass filter with a Canberra-Packard (Meriden, CT, USA) Cell harvester. ^3H -thymidine incorporation was detected on a Matrix 96 beta counter from Canberra-Packard.

For all subjects, results were expressed as mean ^3H -thymidine incorporation \pm SEM of six replicate wells at each passage. Results were transformed in all conditions tested as an average of percentage of control for each subject according to this formula:

[%proliferation = (average ^3H -thymidine incorporation for stimulation minus average ^3H -thymidine incorporation for control) divided by average ^3H -thymidine incorporation for control].

The optimal concentrations of dexamethasone, PDGF-BB and TGF- β 1 were determined from dose response curves (data not shown). Optimal concentrations of ET-1 for DNA and collagen synthesis were

obtained from previous reports of similar experiments (Battistini *et al.* 1993).

Procollagen I and III measurement

Fibroblasts were cultured and prepared as described for DNA synthesis (Dubé *et al.* 1998) except that 1.5×10^5 cells were seeded in 24 well plates in DME with 0.5% FCS and incubated for 12 h at 37 °C and 5% CO_2 . After this initial period, medium was removed and replaced by different culture media for another 24 h: (1) DME containing 0.5% FCS alone; (2) with addition of 10 nM of ET-1; (3) with addition of 10 nM of ET-1, 5 ng/ml of PDGF-BB, 5 ng/ml of TGF- β 1 and in the presence or absence of 10^{-7} M dexamethasone. At the end of this period, the stimulation medium was removed and replaced by freshly prepared pulsing medium (DME containing 0.1 mM of ascorbate). After another 24 h, the pulsing medium was collected for each of the six duplicate conditions and frozen at -80 °C until use. Cells were trypsinized, centrifuged, and resuspended in medium for an haematocytometer count of each well. Procollagen I and III (500g) were measured by RIA according to instructions supplied by the supplier (Pharmacia and Upjohn, Don Mills, Ontario, Canada). Results were obtained in ng of procollagen/ 10^5 fibroblasts and were expressed for all conditions tested as an average of percentage of control for each subject \pm SEM.

Statistical analysis

In order to compare asthmatic to normal subjects for different experimental conditions we used a multifactor analysis of variance, where the group, the condition and the effects of dexamethasone were evaluated. For each subject and group, we first compared the effect of dexamethasone on all of the eight conditions. The advantage of this design was that sources of variability between subjects were eliminated from the experimental errors; only variations within subjects were considered. An interaction factor was included between dexamethasone and the other conditions. To support the validity of the performed tests, normality and variance assumptions were verified and no evidence to reject the null hypothesis was encountered. When interactions were statistically significant, separate analyses were performed using Student's paired *t*-test. Data were analysed using the statistical package SAS (SAS Institute Inc., Cary, NC, USA).

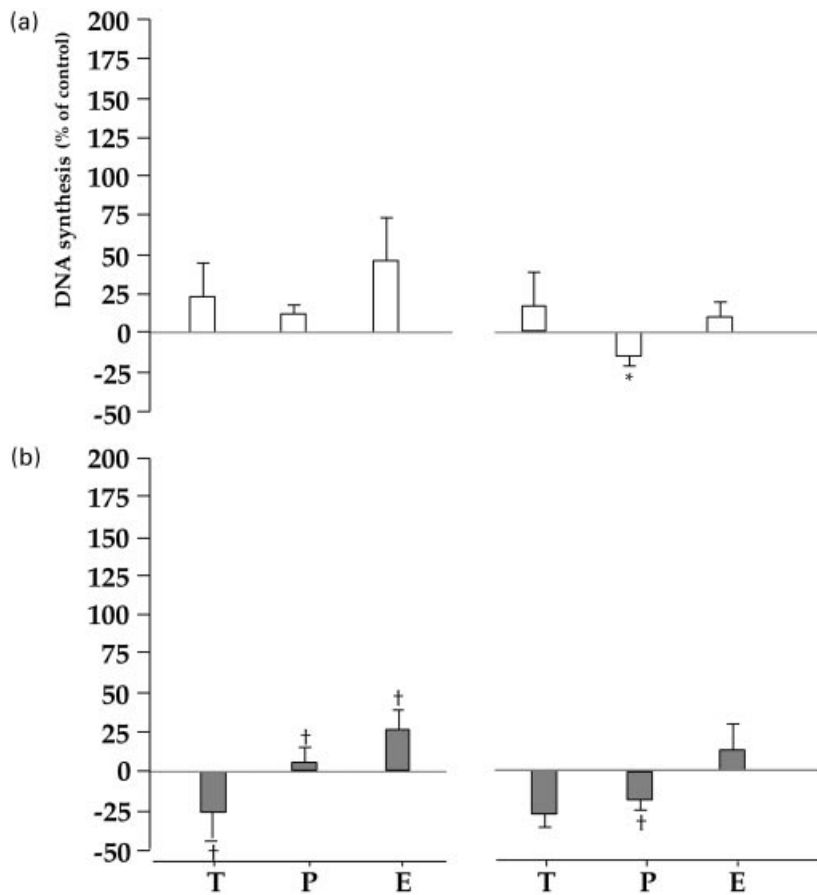


Figure 1. Effect of TGF- β 1 (T), PDGF-BB (P) or endothelin-1 (E) in absence (a) or presence of dexamethasone (b) on bronchial fibroblast DNA synthesis by ^3H -thymidine incorporation. Results are expressed as percentage of control value \pm SEM of the mean of six replicate wells for each condition. Statistical significance is indicated by asterisk ($P < 0.05$ when compared to control) and cross ($P < 0.05$ when compared to similar conditions without dexamethasone).

Results

Proliferative response of bronchial fibroblasts to ET-1, PDGF-BB and TGF- β 1

Cells were assayed for DNA synthesis in the presence of chosen cytokines alone (Fig. 1a) or in combinations (Fig. 2a). TGF- β 1 had no effect on ^3H -thymidine incorporation by both normal and asthmatic fibroblasts. PDGF-BB significantly decreased ^3H -thymidine incorporation in asthmatic fibroblasts ($P < 0.05$), but had no effect on normal fibroblasts. ET-1 had no effect on bronchial fibroblasts of asthmatic or normal subjects. The combination of PDGF-BB and TGF- β 1 had no effect on bronchial fibroblasts of both normal and asthmatic subjects (Fig. 2a). Similar responses were also obtained with the combination of ET-1 and TGF- β 1. The combination of ET-1 and PDGF-BB increased DNA synthesis of normal subjects' bronchial fibroblasts ($P < 0.05$). When ET-1, PDGF-BB and TGF- β 1 were added to the incubation media, a significant increase in DNA synthesis was observed in both groups. The

proliferation of normal subjects' bronchial fibroblasts increased to 131.4% ($P < 0.05$) above control and that of asthmatics' cells to 124.1% ($P < 0.05$) above control.

Procollagen I and III modulation by ET-1 alone or a combination of ET-1, PDGF-BB and TGF- β 1

Having found that the combination of ET-1, PDGF-BB and TGF- β 1 was highly mitogenic and that ET-1 alone had no mitogenic activity for bronchial fibroblasts, we evaluated the possibility that these mediators could modulate cell procollagen synthesis (Fig. 3a). ET-1 alone significantly increased procollagen I synthesis of asthmatic subjects' bronchial fibroblasts ($P < 0.05$) but not of normal cells. The combination of ET-1, PDGF-BB and TGF- β 1 significantly increased procollagen I synthesis of asthmatic subjects' bronchial fibroblasts ($P < 0.05$) but not of normal bronchial fibroblasts. ET-1 alone or in combination with PDGF-BB and TGF- β 1 had no effect on procollagen III synthesis of bronchial fibroblasts from asthmatic or normal subjects.

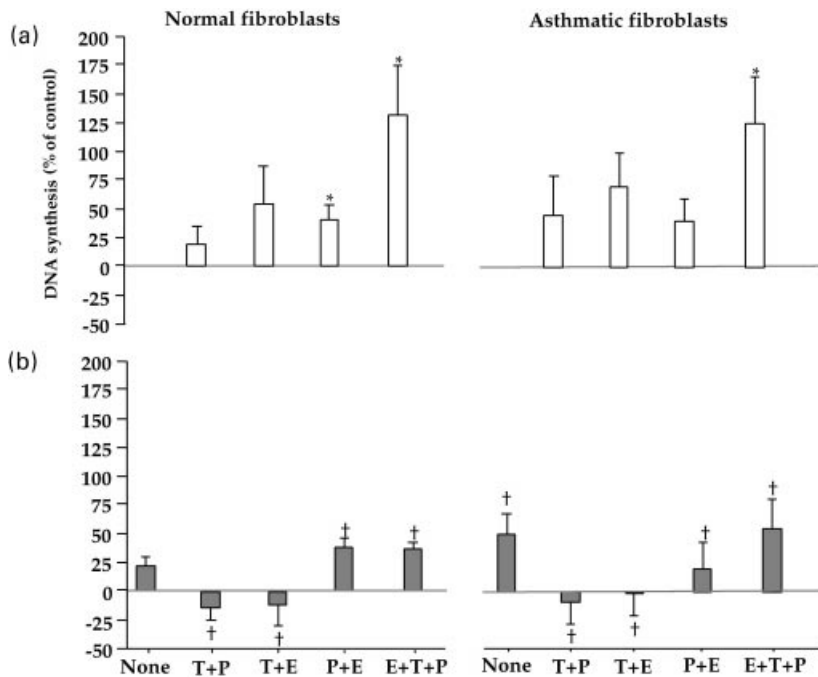


Figure 2. Effect of TGF- β 1 (T), PDGF-BB (P) and endothelin-1 (E) in combination and in absence (a) or presence of dexamethasone (b) on bronchial fibroblast DNA synthesis by ^3H -thymidine incorporation. Results are expressed as percentage of control value \pm SEM of the mean of six replicate wells for each condition. Statistical significance is indicated by asterisk ($P < 0.05$ when compared to control) and cross ($P < 0.05$ when compared to similar conditions without dexamethasone). None: condition without cytokines but with dexamethasone.

Effect of dexamethasone on bronchial fibroblast DNA synthesis and procollagen production

Dexamethasone (10^{-7} M) alone increased the DNA synthesis of asthmatic subjects' bronchial fibroblast ($P < 0.05$) without any significant effect on normal subjects' cells when compared to control condition (Fig. 2b). A decrease in DNA synthesis of bronchial fibroblasts was observed when dexamethasone was added simultaneously to the individual cytokines tested above with the exception of ET-1 effect on asthmatic subjects' bronchial fibroblasts (Fig. 1b). A decrease in DNA synthesis of bronchial fibroblasts was also observed when cells were incubated simultaneously with dexamethasone and different combinations of cytokines (Fig. 2b). Dexamethasone alone or added simultaneously to each condition did not modify the procollagen synthesis of bronchial fibroblasts from either normal or asthmatic subjects, except for the increase in collagen synthesis (282.4% of control, $P = 0.028$ by Wilcoxon's sign test) of the ET-1, PDGF-BB and TGF- β 1 stimulated asthmatic subjects' bronchial fibroblasts (Fig. 3b).

Discussion

Airway subepithelial fibrosis has been shown to be associated with airway hyperresponsiveness (Boulet *et al.* 1997). However, the specific cytokines or

mediators leading to the activation of the airway repair process in asthma have not been clearly identified. To our knowledge, this is the first study which directly addresses the potential role of ET-1 as a modulator of proliferation and of the collagen synthesis by human bronchial fibroblasts isolated from normal and asthmatic subjects. Our results show that the combination of ET-1, PDGF-BB and TGF- β 1, but not these mediators alone or in double combination, increased DNA synthesis of bronchial fibroblasts from normal and asthmatic subjects *in vitro* in a way that could explain, at least partially, the collagen deposition observed in asthma. Dexamethasone decreased the proliferative response triggered by the combination of cytokines/mediators of normal and asthmatic subjects' bronchial fibroblasts. Furthermore, the same combination increased collagen I synthesis by asthmatic subjects' bronchial fibroblasts *in vitro*. Interestingly, when dexamethasone was added simultaneously to the ET-1, PDGF-BB and TGF- β 1 combination, collagen I synthesis of the asthmatic subjects' bronchial fibroblasts was further increased *in vitro*.

Pulmonary fibrosis has been associated with fast-growing clones of lung fibroblasts, even in the absence of any stimuli (Jordana *et al.* 1988). On the contrary, keloid-type fibrosis has been associated with an increased baseline capacity to produce collagen in the absence of fast-growing fibroblasts (Bettinger *et al.* 1996). We previously showed that asthmatic subjects' bronchial fibroblasts had a decreased baseline DNA

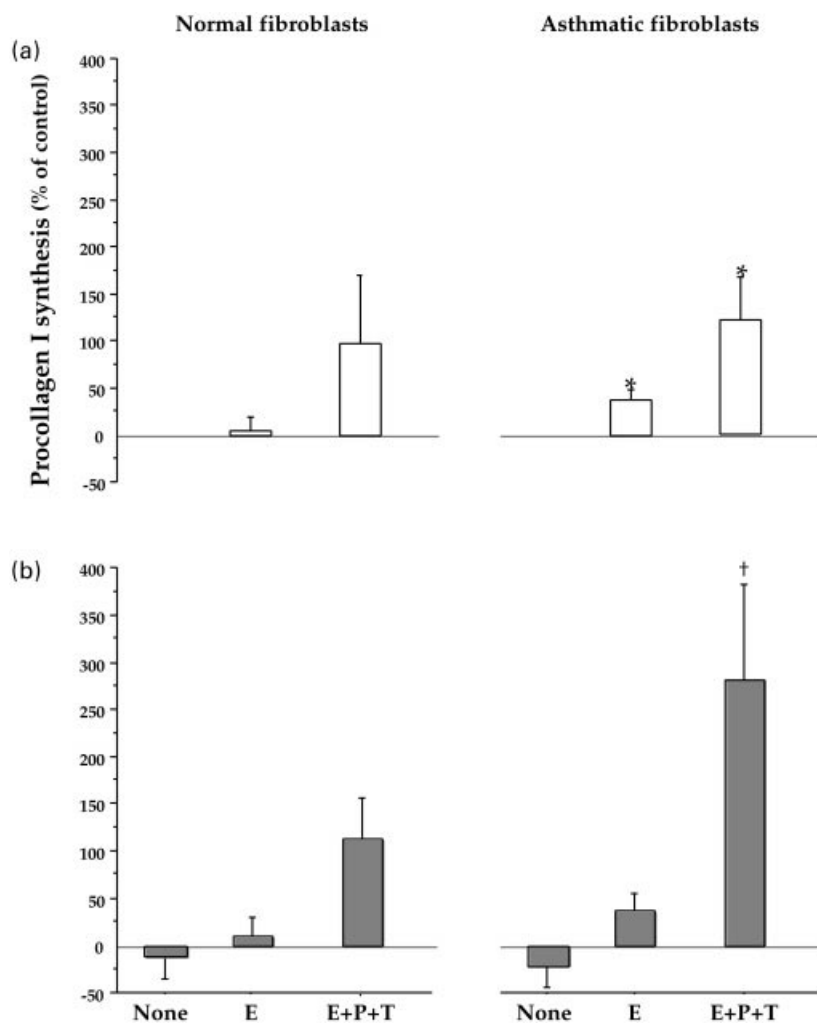


Figure 3. Effect of ET-1 (E) alone or in combination with TGF- β 1 (T) and PDGF-BB (P) in absence (a) or presence of dexamethasone (b) on bronchial fibroblast procollagen I synthesis by RIA. Results are expressed as percentage of control value \pm SEM of the mean duplicate wells for each condition. Statistical significance is indicated by asterisk ($P < 0.05$ when compared to control) and cross ($P = 0.028$ by Wilcoxon's sign test when compared to similar conditions without dexamethasone). None: condition without cytokines but with dexamethasone.

synthesis and presented a baseline collagen synthesis similar to that of bronchial fibroblasts from normal subjects (Dubé *et al.* 1998). This led us to believe that a combination of mediators released following various stimuli such as allergic exposure (Gizycki *et al.* 1997) could activate bronchial fibroblasts to increase their DNA synthesis and their collagen synthesis in a manner which may account for the asthmatic subepithelial fibrosis.

In repair and fibrotic states, TGF- β can either stimulate or inhibit fibroblast DNA synthesis and enhance extracellular matrix deposition by human fibroblasts isolated from different anatomic sites (Fine & Golstein 1986; Bryckaert *et al.* 1988; Thorton *et al.* 1990; Raghow 1991; Story *et al.* 1993; Border & Noble 1994). However, our results do not support a major role for TGF- β 1 alone in the activation of bronchial fibroblasts.

PDGF is recognized to potentiate DNA synthesis and extracellular matrix deposition of fibroblasts from the bone marrow and the lung (Bryckaert *et al.* 1988; Thorton *et al.* 1990). However, while it slightly decreased ^3H -thymidine incorporation of asthmatic subjects' bronchial fibroblasts ($P < 0.05$) it had no effect on normal bronchial fibroblasts.

ET-1 is implicated in airway wall remodelling and fibroblast proliferation (Cambrey *et al.* 1994; Hay *et al.* 1996). Our data demonstrated however, that ET-1 alone had no effect on bronchial fibroblast DNA synthesis but increased procollagen I synthesis of asthmatic subjects' bronchial fibroblasts (38.2% over control, $P < 0.05$). Furthermore, in our experiments the triple combination of TGF- β 1, PDGF-BB and ET-1 was necessary to increase DNA synthesis of bronchial fibroblasts from normal and asthmatic subjects *in vitro*. These observations show that the synergistic effect of the mediators

seems to be needed to activate bronchial fibroblasts proliferation and procollagen I synthesis in asthmatic subjects *in vitro*.

ET-1 interacts with specific receptors on the cell surface which activate postreceptor mechanisms leading to a biological response (Battistini *et al.* 1993). Such receptors have been described on Swiss 3T3 fibroblasts and on different human cell types (Battistini *et al.* 1993). Our experiments show that bronchial fibroblasts respond to ET-1 at least in combination with other cytokines/mediators, and this suggests the presence of such receptors on bronchial fibroblasts.

Glucocorticoids, the most effective medication used in long-term treatment of asthma, modulate many fibroblast functions, including collagen synthesis and proliferation. Some reports showed that corticosteroids increased the *in vitro* proliferation of human fibroblasts of different origins (Kondo *et al.* 1985; Sano *et al.* 1988; Conover *et al.* 1995), whereas others reported a decrease in proliferation (Durant *et al.* 1986; Görmär *et al.* 1990; Hein *et al.* 1994). Our observations show that dexamethasone alone *in vitro* increases DNA synthesis of asthmatic subjects' bronchial fibroblasts without having any effect on those from normal subjects. However, when dexamethasone is added simultaneously to the cytokines/mediators in any of the conditions above, it decreases the DNA synthesis of bronchial fibroblasts. Furthermore, the increase in DNA synthesis induced by the combination of ET-1, PDGF-BB and TGF- β 1 on normal and asthmatic subjects' bronchial fibroblasts was not observed in the presence of dexamethasone. These data suggest that dexamethasone interferes with postreceptor activation mechanisms of these mediators and that it has a differential effect on fibroblasts depending on their state of activation.

Most of the data supporting a reduction in collagen synthesis by fibroblasts with corticosteroids come from studies carried out with skin fibroblasts or from clinical and pathological observations on skin atrophy in patients using topical corticosteroids (Görmär *et al.* 1990; Haapasaari *et al.* 1997). Studies comparing human skin fibroblasts to human colonic fibroblasts have however, shown significant differences in the collagen synthesis response to corticosteroids (Martens *et al.* 1992). In our study, dexamethasone increased further the synthesis of procollagen I observed *in vitro* in response to a combination of ET-1, PDGF-BB and TGF- β 1. The relevance of these observations *in vivo* has not yet been defined but they suggest that dexamethasone could initially induce a transient collagen synthesis, i.e. until the levels of ET-1 and TGF- β are downregulated by

corticosteroids. Our results suggest that the effect of corticosteroids *in vivo* may be secondary to a decrease in inflammatory mediators and fibroblast proliferation rather than decreasing collagen synthesis. To assess a fibrotic state, it is necessary to understand the complex equilibrium between the pro-fibrotic mechanisms, i.e. the number of effector cells (fibroblasts) and the quantity of collagen they synthesize compared to the antifibrotic mechanisms which include collagen phagocytosis by fibroblasts and the enzymatic degradation system of MMP-9 and TIMP-1. Although in our study, corticosteroids did not decrease directly collagen synthesis, we can hypothesize that they increase collagenase synthesis and activity by a decrease in TIMP-1. Further studies should be undertaken to better understand the mechanisms leading to collagen accumulation in the airways. Future experiments evaluating collagenase and TIMP-1 expression with the same stimuli will provide other elements for evaluating the extent of these differences and should provide more insight into the mechanisms of subepithelial fibrosis in asthma.

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